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NEUROPHARMACOLOGICAL CHARACTERIZATION OF BOTULINUM NEUROTOXIN

MIDTERM REPORT

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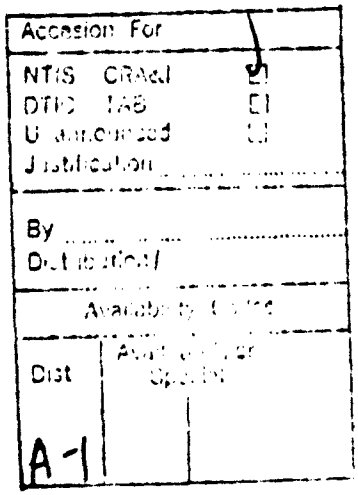
TABLE OF CONTENTS



FOREWORD

In conducting the research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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I. INTRODUCTION

The purpose of the work is to find drugs that will antagonize the actions of clostridial neurotoxins. Studies will be conducted initially on neuromuscular preparations, cells in culture, or other tissues obtained from laboratory animals. When a promising drug is identified, it will be tested as an antagonist of toxin action on human tissues.

During the first half of the contract, experiments have been done to evaluate Zaprinast and other drugs that alter the levels or tissue distribution of c-GMP and its synthesizing and degrading enzymes. These substances have previously been reported to antagonize clostridial neurotoxins on a tissue culture line (PC-12; Sandberg et al.,J. Neurosci. 9:3946-3954, 1989). The results have produced an unexpected outcome. The drugs antagonize toxins on one type of tissue preparation but not another. This unexpected finding may have important implications for understanding toxin action.

In addition, experiments have been done to try to identify a "universal antagonist" of clostridial neurotoxins. In the present context, universal antagonist means a drug that will delay the onset of effect or hasten the rate of recovery from the poisoning due to botulinum neurotoxin and tetanus toxin. This could be due to an action that antagonizes binding, antagonizes internalization, or antagonizes intracellular expression of toxicity; it could also be due to an action that reverses intracellular poisoning. As noted above, initial work will be done on animal tissues but any promising candidate as an antagonist will also be studied on human tissues.

II. METHODS

Toxins and reagents. Tetanus toxin was purchased from Calbiochem and from the Massachusetts Public Health Biological Laboratories. Botulinum neurotoxin type A was isolated in the laboratories at Jefferson.

In vitro bioassay. Phrenic nerve-hemidiaphragm preparations were excised from mice and placed either in a tissue bath or an incubation bath (see Results). Tissues were suspended in a physiological solution that was bubbled with 95% O_2, 5% CO_2. The solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; Na_2HPO_4, 1.0; glucose 11. Solutions were supplemmented with gelatin to diminish adsorption and nonspecific inactivation of toxin. The tissue baths were kept at 35° C and the incubation tubes were kept at 4° C.

The parameters of phrenic nerve stimulation were 0.2 Hz square waves of 0.1 to 0.3 msec. duration. Muscle twitch was recorded with a force-displacement transducer connected to a physiological recorder. Toxin-induced paralysis of neuromuscular transmission was measured as a 90% reduction in muscle twitch amplitude evoked by nerve stimulation.

III. RESULTS

A. Zaprinast and Related Compounds

Background. In the recent past, Dr. Terry Rogers and his associates have described what appears to be a remarkable interaction between Zaprinast, an inhibitor of c-GMP degrading phosphodiesterases, and tetanus toxin (Sandberg et al., see above). When studied on PC-12 cells, tetanus toxin produced concentration-dependent blockade of ^{3}H-acetylcholine release, and it simultaneously produced depression in tissue levels of c-GMP. These findings led Sandberg et al. to speculate that the toxin

depressed levels of c-GMP as a primary effect, and it then caused blockade of exocytosis as a secondary effect. To test this idea, Sandberg et al. evaluated a number of c-GMP analogues to determine whether they could antagonize toxin induced blockade. They found that c-GMP analogues such as 8-bromo-c-GMP were very effective in overcoming blockade of ^{3}H-acetylcholine release, but analogues of c-AMP (i.e., 8-bromo-c-AMP) and other cyclic nucleotides were not effective.

As part of this effort, the University of Maryland group also tested Zaprinast, an inhibitor of phosphodiesterases that degrade c-GMP. This compound was found to be very active. When tested at levels of 100 nM, Zaprinast completely reversed the poisoning due to tetanus toxin.

Because of the potential importance of these findings, the Principal Investigator invited Dr. Rogers to visit Jefferson. This was part of an agreement to initiate a series of collaborative experiments. The premise of the agreement was that the Principal Investigator would test on neuromuscular preparations the same drugs that Dr. Rogers had tested on PC-12 cells. In addition, the work at Jefferson would include botulinum neurotoxin as well as tetanus toxin. The present report provides the initial results from this work.

Current Findings. The effects of 8-bromo-analogues and Zaprinast were examined on cholinergic transmission at the mouse phrenic nerve-hemidiaphragm. All drugs were found to have little or no effect on muscle responses evoked indirectly by nerve stimulation or directly by potassium depolarization.

The effects of botulinum neurotoxin and tetanus toxin on neuromuscular transmission have already been reported by numerous investigators, including the author. As discussed above, Sandberg et al. studied the effects of tetanus toxin on ^{3}H-acetylcholine release from PC-12 cells. It should be noted that there was one unusual aspect to the reported dose-response data. Sandberg et al. found that 1 nM

toxin did not block exocytosis within 3 hours, but at concentrations of 5 to 10 nM it produced a maximal effect. This finding indicates that : i.) the dose-response curve is extraordinarily steep, and ii.) the PC-12 preparation is less responsive to the blocking action of tetanus toxin than the neuromuscular junction. The latter may be important to remember, because the neuromuscular junction is itself somewhat unresponsive to tetanus toxin.

In an effort to duplicate the drug antagonism data of the Maryland group, the author studied the interaction between 8-bromo-analogues, Zaprinast and clostridial neurotoxins. The putative antagonist was added to tissues 15 minutes before botulinum neurotoxin type A (1×10^{-11}M) or tetanus toxin (1×10^{-9}M). The resulting paralysis times were monitored and the results are given in Table 1. The data show that the putative antagonists did not alter the course of toxin-induced neuromuscular blockade.

The work was broadened to consider two phospholipase A2 neurotoxins, beta bungarotoxin and crotoxin. The experimental paradigm was identical to that described for clostridial toxins, and the outcome was the same. As shown in Table 2, the drugs did not delay the onset of poisoning due to snake neurotoxins.

These results are clearly different from those obtained by Sandberg, et al. Assuming that the originally reported antagonism is correct, one must propose an explanation. An interesting possibility is that the mechanisms that govern acetylcholine release from PC-12 cells and from the neuromuscular junction are different, but clostridial toxins can block exocytosis at both sites. If exocytosis in PC-12 cells is regulated by c-GMP, then the results of Sandberg et al. would seem reasonable; and if transmitter release from the neuromuscular junction is governed by non-c-GMP mechanisms, then the present results are appropriate.

B. Lectins

Background. The current work is part of a collaborative effort both to isolate the botulinum neurotoxin receptor and to identify drugs that will antagonize toxin binding to these receptors. This collaborative effort is seeking to determine whether the receptor might be a glycoprotein. Part of the approach to addressing this question has been to test whether lectins that have affinity for known carbohydrate residues behave as toxin antagonists. Two tests for antagonism have been employed: i.) a ligand binding assay using iodinated toxin and brain membrane preparations (DAMD17-86-C-6161) and ii.) an in vitro bioassay using the mouse phrenic nerve-hemidiaphragm preparation.

Current Findings. Eight lectins have been tested for their abilities to antagonize clostridial neurotoxin binding and activity. The lectins (and the carbohydrates for which they have affinity) are: i.) Anguilla anguilla (α-fucose), ii.) Atrocarpus integrifolia (α-galactose), iii.) Datura stramonium (N-acetyl-β-glucosamine), iv.) Glycine max (N-acetyl-α-galactosamine), v.) Canavalia ensiformis (α-mannose, α-glucose, N-acetylglucosamine), vi.) Ricinus communis (β-galactose, N-acetyl-β-galactosamine), vii.)Limax flavus (N-acetylsialic acid) and viii.) Triticum vulgaris (N-acetyl-glucosamine, N-acetylsialic acid).

Of this group, only two provided significant protection against toxins in an in vitro assay and at reasonable concentrations. These lectins were Limax flavus and Triticum vulgaris, implicating an N-acetylsialic acid or N-acetylglucosamine as putative components of a receptor. However, the fact that Canavalia ensiformis did not provide significant protection seems to eliminate N-acetylglucosamine as the relevant moiety.

The original experiments with lectins and toxins were done with only one serotype. When it was discovered that Triticum vulgaris and Limax flavus were antagonists of type B toxin, two follow-up experiments were done. First, a full dose-response curve was generated to demonstrate the effectiveness of the lectin in

antagonizing type B toxin (Table 3). Next, a single concentration of lectin was tested as an antagonist of all clostridial toxins (Table 4).

When TVL was added to phrenic nerve-hemidiaphragm preparations simultaneously with botulinum neurotoxin type B (7×10^{-12} M), the lectin produced concentration-dependent antagonism of the onset of toxin-induced paralysis. At a concentration of 3×10^{-5} M, TVL produced a highly statistically significant effect ($p < 0.001$). When this same concentration of lectin was tested against neurotoxin types A, C, D, E and F, as well as tetanus toxin, it produced significant antagonism in all cases. By contrast, the lectin did not protect tissues aginst the neuromuscular blocking effects of a snake neurotoxin (β-bungarotoxin).

A series of experiments were performed to confirm that TVL was antagonizing clostridial toxins by virtue of blocking the receptor. For this purpose, tissues were incubated at 4°C during exposure to toxin and/or lectin. Incubation at low temperature allows binding to proceed, but it prevents the subsequent step of internalization. After incubation at 4°C (and see below), tissues were washed and transferred to baths at 35°C. Phrenic nerves were stimulated and the rate of toxin-induced paralysis was monitored.

In the first experiment, tissues were incubated with or without TVL (3×10^{-5} M) for 30 minutes, after which botulinum neurotoxin type B (7×10^{-12} M) was added and tissues were incubated for an additional 60 minutes. Tissues were then washed and suspended in baths, and paralysis time (90%) was monitored. The paralysis times of tissues exposed only to toxin was 90±5 min; the paralysis times of tissues exposed first to lectin and then to toxin was > 200 min.

In the second experiment, the order of addition was reversed. Tissues were incubated with or without botulinum neurotoxin for 30 minutes, after which TVL was added and tissues were incubated for another 60 minutes. In this case, the paralysis

time of tissues exposed only to toxin was 98±6 min and this was closely similar to the paralysis times of tissues exposed first to toxin and then to lectin.

In the final experiment, a somewhat different approach was used. TVL was incubated with or without N-acetyl-β-glucosamine (1×10^{-1} M) for 30 min. This mixture was then incubated with tissues for 30 min at 4°C, after which botulinum neurotoxin was added and incubation was continued for another 60 minutes. After being washed, tissues were suspended in baths and paralysis times were monitored. A control group of tissues that was incubated only with toxin had a paralysis time of 100±11 min. The tissues incubated with lectin in the absence of carbohydrate had a paralysis time of >200 min; tissues incubated with lectin plus carbohydrate had a paralysis time of 121±12 min.

The data indicate that TVL is not itself toxic, that TVL antagonizes botulinum toxin at the binding step, and that TVL previously exposed to a carbohydrate for which it has affinity ceases to antagonize toxin.

C. Ongoing Studies

A decision has been made to develop three systems that will permit the study of toxin action on human tissues, or on model cells that have been transformed to express human receptors. These three systems are:

- Surgically excised human neuromuscular junctions

- Co-cultures in which human motoneurons and human striate muscle are maintained under conditions that maximize formation of functional neuromuscular junctions.

- Xenopus oocytes that have been transfected with total RNA or mRNA to express proteins of interest, such as membrane receptors for clostridial toxins.

Regarding human neuromuscular junctions, IRB approval has been obtained to proceed with this project, under the aegis of Dr. Robert Sataloff, the surgeon who will remove the tissue. There are two important points that should be noted about this procedure. First, no surgery is done for the purpose of excising tissue for research. Surgery is done only for clinical indications, and the tissue used is that which would be excised even if no research were being done. Second, the tissue being removed is otolaryngeal in origin, and the thin . ure of the tissue will facilitate research.

At the same time that the IRB approval was being sought, a special apparatus was being built to hold human tissues for electrophysiologic recordings. The apparatus was designed by Dr. Lee Chabala. It consists of a tissue chamber mounted in a Peltier-driven temperature controller, and the chamber is built to sit on a microscope stage. This device will be used . rt of an effort to do extracellular loose patch clamp recordings of endplate currents in the presence and absence of clostridial toxins. The

miniaturized temperature controller will allow for variation in temperatures from 4° C to normal body temperature (~37° C), and this in turn will permit experiments that separate the binding step from subsequent steps in the toxin-induced paralysis.

In relation to co-cultures of human nerve and human muscle, it should be noted that this area of work has just begun. A substantial part of the coming Year will be spent establishing this technique. When co-cultures have been developed, they will be examined electrophysiologically in a manner similar to excised tissues.

The third technique being developed is that of transfecting Xenopus oocytes. Substantial progress has been made in this area. The techniques for excising and maintaining eggs have already been developed. The methods for isolating total RNA and mRNA from rat brain have been acquired, and eggs have been injected. Evidence for transfection has been obtained both biochemically (i.e., ligand binding assays) and functionally (i.e., recording of ion channel activity). The methods that have been developed for laboratory animal work will now be applied to the study of human mRNA.

TABLE 1

Effects of drugs on clostridial
neurotoxin-induced neuromuscular blockade.

	Paralysis Time (Minutes)	
Drug	Botulinum Neurotoxin	Tetanus Toxin
None	126 ± 13	131 ± 9
8-Bromo-cAMP	119 ± 7	121 ± 12
8-Bromo-cGMP	139 ± 11	138 ± 16
Zaprinast	132 ± 14	142 ± 12

TABLE 2

Effects of drugs on phospholipase A2 neurotoxin-induced neuromuscular blockade.

	Paralysis Time (Minutes)	
Drug	β-Bungarotoxin	Crotoxin
None	100 ± 6	131 ± 18
8-Bromo-cAMP	94 ± 3	142 ± 14
8-Bromo-cGMP	103 ± 7	140 ± 11
Zaprinast	100 ± 9	140 ± 13

TABLE 3

Dose-response data for the ability of Triticum vulgaris lectin to antagonize the neuromuscular blocking properties of botulinum neurotoxin type B (1 ng/ml).

Lectin Concentration	90% Paralysis Time (min.)
None	98 ± 8
10^{-6} M	97 ± 3
10^{-5} M	126 ± 8
3×10^{-5} M	206 ± 6
10^{-4} M	246 ± 5
10^{-3} M	>300

TABLE 4

Neuromuscular blockade induced by clostridial toxins in absence and presence of 3×10^{-5} Triticu[illegible]lgaris.

Toxin		Concentration ng/ml	50% Paralysis Time in minutes -lectin	50% Paralysis Time in minutes +lectin
Botulinum Neurotoxin serotype	A	2	30 ± 9	50 ± 10
	B	1	56 ± 10	88 ± 11
	C	5	75 ± 14	95 ± 15
	D	2	25 ± 5	34 ± 6
	E	2	40 ± 6	72 ± 20
	F	8	60 ± 5	95 ± 5
Tetanus Toxin		200	80 ± 15	125 ± 15